

Research Paper

Isolation and characterization of novel thermophilic lipase-secreting bacteria

Mohammed Rabbani¹, Mohammad Reza Bagherinejad¹,
Hamid MirMohammad Sadeghi¹, Ziaedin Samsam Shariat², Zahra Etemadifar³,
Fateme Moazen¹, Manizheh Rahbari³, Ladan Mafakher³, Saeideh Zaghian³

¹Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences,
Isfahan University of Medical Sciences, Isfahan, Iran.

²Department of Biochemistry, School of Pharmacy and Pharmaceutical Sciences,
Isfahan University of Medical Sciences, Isfahan, Iran.

³Department of Biology, School of Basic Sciences, University of Isfahan, Isfahan, Iran.

⁴Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences,
Isfahan University of Medical Sciences, Isfahan, Iran.

Submitted: April 11, 2012; Approved: April 4, 2013.

Abstract

The purpose of the present study was to screen and identify the lipase-producing microorganisms from various regions of Iran. Samples collected from hot spring, Persian Gulf, desert area and oil-contaminated soil, were analyzed for thermophilic extracellular-lipase producing organisms. Six strains with high activity on rhodamine B plates were selected for chemical identification and further study. Among these isolated bacteria, four strains show higher activity in pH-Stat method at 55 °C. These strains were identified by PCR amplification of 16s rRNA genes using universal primers. Fermentation increased the activity up to 50%. The growth medium, designed for lipase production, increased the activity up to 4.55 folds. The crude supernatant of ZR-5 after fermentation and separation the cells, was lyophilized and the activity was measured. Total activity of this strain was 12 kU/g that shows its potential for industrial uses. Further study is required for purification of enzyme and calculation its specific activity. Immobilization is another approach should be considered.

Key words: extracellular lipase, pH-stat method, thermophilic lipase, fermentation.

Introduction

Lipase (triacylglycerol hydrolase, E.C. 3.1.1.3) belongs to serine hydrolase enzymes which can catalyze the hydrolysis and synthesis esters from glycerol and fatty acids. These reactions usually proceed with high chemo-, regio- and/or enantioselectivity at the interface between the insoluble substrate and water (Nguyen *et al.*, 2010). Enzyme-mediated reactions are gradually replacing the traditional and expensive chemical methods. The products of lipase-catalyzed reactions have higher quality and their energy consumption is lower compared to the conventional high-temperature, high-pressure-steam splitting methods (Treichel *et al.*, 2010). Also because of high capability of lipase to perform a specific range of biotransformation,

they are used widely in different industry such as food and dairy, detergent, cosmetic, leather, paper and pulp, bio-diesel and pharmaceutical (Hasan *et al.*, 2007). Among them thermophilic lipases have special characterizations which are more in interest in industrial and chemical process. They show stability and more activity in higher temperature and they are usually stable in the presence of chemicals (Castro-Ochoa *et al.*, 2005; Messias *et al.*, 2009; Uttatree *et al.*, 2010). High global demand for lipases and billion-dollar business (more than 1000 ton each year which makes lipases third largest group of enzymes based on total sales volume after protease and carbohydrase) has resulted in increased number of research to identify, isolate and introduce new lipase-producing microorganisms (Hasan *et al.*, 2006; Shu *et al.*, 2010; Treichel *et al.*, 2010).

Lipases are produced by animals, plants and various microorganisms and many of them secrete the enzyme in extracellular spaces. However due to specificity of action, microbial lipase have been the focus of attention especially for extracellular lipases (Sheikh Abdul Hamid *et al.*, 2003; Yuan *et al.*, 2010). The majority of lipases that are currently used in industry have microbial sources (Castro-Ochoa *et al.*, 2005; Hasan *et al.*, 2006; Nthangeni *et al.*, 2001; Adiguzel *et al.*, 2009). Some of the advantages of these microbial lipases include: high and wide range conditions stability, simplicity and ease of mass production and gene manipulation, activity in extreme conditions (high/low temperature and pH), no requirement for cofactors, high specificity and low waste production, the possibility of use in continuous operation, ease of recovery and reuse, cost benefit and low required downstream processing (Haba *et al.*, 2000; Sheikh Abdul Hamid *et al.*, 2003; Treichel *et al.*, 2010).

One of the richest sources for identification and isolation of new strain of microorganisms is soil. Such studies can introduce new lipases with stability in different temperature and pH, specificity for certain fatty acids and substrates and enantioselectivity. The purpose of the present study was to screen and identify the lipase-producing microorganisms from desert region of Iran and Persian Gulf.

Materials and Methods:

Sample collection and bacterial strain isolation

Soil samples were collected from various regions of Iran including the oil fields and Persian Gulf in the south, desert area in the center and the hot spring around Isfahan province. Soil samples were sieved through a 2 mm mesh to remove plant debris and soil particles. After drying the samples at room temperature (20 °C) for 24 h, they were stored at 4 °C.

For isolation of microorganisms, 10 g of samples were suspended in 90 mL of sterile saline and incubated on a shaker incubator at 80 °C for 30 min. Ten-fold serial dilution method in normal saline was used to dilute the samples after cooling the suspension in room temperature. Sterile agar plate was inoculated with 100 µL of each dilution tube and incubated at 37 °C for 24 h to obtain isolated colonies. Later, each colony was selected and sub-cultured on nutrient agar plates for isolation and purification (Rabbani *et al.*, 2009b; Rabbani *et al.*, 2009a).

Screening of strains for extracellular lipase

Rhodamine B plate assay was used for screening of lipolytic activity of isolated strains according to method of Kouker and Jaeger, 1987, with some modifications. The rhodamine B plate contained 0.8% w/v nutrient broth, 0.4% w/v NaCl, 500 µL of 0.01% Rhodamine B solution, 1% w/v agar and 7.5% v/v olive oil. The pH of the medium was adjusted to 7.0. An overnight fresh colony of isolated strains

was sown on a rhodamine B plate and incubated at 37 °C for 24 h. After incubation, the plates were exposed to UV light (350 nm) for determination the lipase activity of the isolated strains. The orange fluorescence of plates and its intensity was used as an index for lipolytic activity. The results were quantified from 1-4 according to this index. Colonies with no fluorescence have no lipase activity. The colonies with high fluorescence were selected for further studies and quantitative lipase activity assay by titration (pH-Stat method).

Strains identification

Biochemical identification

Biochemical tests were also used for identification of isolated lipase positive colonies. According to the Bergey's manual of systemic bacteriology, these tests were selected and were performed in triplicate (Claus and Berkeley, 2011). The tests included: Gram staining reaction, spore position and shape, swelling of sporangia, aerobic or anaerobic growth, Vogues-Proskauer test, oxidase, catalase, citrate consuming, nitrate reductase and lecithinase reaction. The cell morphology was examined by light microscopy and biochemical characteristics were investigated at room temperature and 37 °C. Growth under anaerobic conditions was checked by inoculating a trypton soy broth with the isolated strains and incubating in an anaerobic jar supplemented with a gas pack strip type A for several days.

Thermophilic characterization

Thermophilic characterizations were analyzed at two levels. First, isolated organisms were cultured in high temperature (50 °C) and the thermophilic property of these isolated strains was established. The thermophilic activity of lipases from isolated strains was assayed in the second level by monitoring the lipase activity in 55 °C for 10 min using pH-Stat method.

Molecular identification

PCR amplifications of the 16S rRNA genes were performed with cell lysates obtained using a micro homogenizing system (beads cell disrupter, Micro Smash MS-100). Cells in exponential growth phase (OD 0.6 at 600 nm) were collected by centrifugation (3500 g, 10 min) and resuspended in PBS (pH 7.2). The cells (500 µL cell suspension) were disrupted using 0.1 g beads class Φ 0.6 at 4000 g for 30 s. The extract was centrifuged (5000 rpm, 10 min) to remove cell debris.

The 16S rRNA genes were amplified (MyCycler™, BioRad) using the universal primers 5'-AACTGGAGGAAGGTGGGGAT-3' and 5'-AGGAGGTGATCCAACCGCA-3'. The amplification reaction contained 1 µL of each primer (25 µM), 0.5 µL of dNTP (10 mM), PCR buffer 2.5 µL, 0.7 µL of MgCl₂ (50 mM), 1 µL of template DNA, 1.25 µL DMSO, 0.5 µL

taq DNA polymerase (Cinnagen, Iran) and made up to 25 μ L with deionized H₂O. The following conditions were used in the amplification of 16S rRNA genes: 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, with final 10 min extension at 72 °C. The PCR products were run on agarose gel and later isolated for sequencing. The 16 S rRNA gene sequence was blasted in NCBI.

Quantitative lipase activity assay

pH-Stat method using an auto-titrator (Titrande 902, Metrohm, Switzerland) was applied for quantitative measurement of thermophilic extracellular lipase activity of isolated strains at 55 °C. The substrate stock emulsion contains 25 mL olive oil and 75 mL Arabic gum solution (5% w/v) which was sonicated continuously for 5 min at 200 watts (UIS250L, Hielscher Sonicator, Germany). Sonication was repeated until there was no more oil sitting at the top of the emulsion. The substrate stock emulsion was diluted 1 part by 1 part of deionized distilled water and used as fresh final substrate emulsion in reaction vessel. After pretitration of substrate emulsion with NaOH 0.01 M, the lipase sample (100 μ L) was added and the reaction started at 55 °C. The end point of program was adjusted at pH 8. The program was monitored for 10 min. At the end, NaOH consumption represents the lipase activity of samples.

Cell cultures

Shake flask culture

Shake flask culture was carried out in 250 mL Erlenmeyer containing 50 mL Luria-Bertani (LB) with 2% olive oil. The flasks were inoculated with 10% seed culture and incubated at 37 °C and 220 rpm for 24 h. The time-course growth curve of isolated organisms was plotted to 8 hour after incubation.

Bioreactor culture

Batch fermentation was performed using two different media in bioreactor containing 1 L media. The primary medium consisted of Luria-Bertani (LB) plus 2% olive oil and the second was designed according to the literature review and previous works, containing NaCl 0.1 g/L, CaCl₂ 0.1 g/L, olive oil 2% V/V, MgSO₄ 0.5 g/L, K₂HPO₄ 0.5 g/L, KH₂PO₄ 0.5 g/L, (NH₄)₂SO₄ 2 g/L and 100 μ L vitamin and trace elements solution. The bacterial isolates were culture using fermentor (BioTron, Korea) at 37 °C, 220 rpm. The reactor was inoculated with 10% seed culture in log phase and fermented for appreciate time according to type of medium. The thermophilic lipase activity was measured at different time after incubation.

Lyophilization

In this step, the culture medium of ZR-5 at optimal time (30 h after incubation) was centrifuged (7000 g, 4 °C) and the supernatant was frozen at -70 and lyophilized at

-85 °C, 0.001 mBar for 48 h using a bench-top lyophilizer (Alpha-2-4 LD Plus, Christ, Germany) and total thermophilic lipase activity of lyophilized powder was measured according to pH-Stat method at 55 °C.

Electrophoresis and zymogram

Native polyacrylamide gel electrophoresis with 10% separating gel and 4% stacking gel was used for protein analysis. At the same time two gels was run for a sample. One of them was stained by Coomassie Blue and the other was used for zymogram. After electrophoresis the gel was placed onto an agar plate containing 0.001% rhodamine B and 3% tributyrin and incubated for 24 h at 40 °C. The lipase band is visualized by a clear zone around the related fluorescent band (350 nm). The molecular weight of the lipase is estimated by comparison this band with standard molecular weight in ladder lane (Kouker and Jaeger, 1987).

Results and Discussion

Isolation and characterization of bacterial isolates

The lipase producing bacteria were isolated from different soil samples. From the total of 63 samples, the Rhodamine negative strains were put aside and the remaining isolates (6 strains) were biochemically characterized using Bergey's Manual of Systemic Bacteriology (Claus and Berkeley, 2011). Based on various biochemical testes shown in Table 1, three bacterial isolates (ZR-1, ZR-5 and WW) were identified as genus *Bacillus*. Time course-growth curve of four strains (ZR-1, ZR-5, PG-1 and WW) with higher thermophilic lipase activity in pH-Stat method is shown in Figure 1.

All the isolated strains with positive rhodamine test were characterized first by conventional biochemical techniques and were further characterized by PCR amplification of 16S rRNA gene. 370 bp of 16S rRNA gene was amplified by PCR for all 4 bacterial isolates (Figure 2). The result sequence was BLAST in NCBI gene bank (<http://www.ncbi.nlm.nih.gov/BLAST>). Blast analysis indicated that, the strain ZR-1 belongs to genus *Bacillus subtilis* (97% homology), strain ZR-5 belongs to genus *Bacillus pumilus* (99% homology), strain PG-1 belongs to *Staphylococcus haemolyticus* (91% homology), and the strain WW belongs to *Bacillus safenis* (100% homology). Because of highest lipase activity in ZR-5, its sequence of 16s rRNA gene of was submitted in NCBI with accession number JN968462.1.

Lipase activity assay

Six strains of bacteria that showed positive results on Rhodamine test (ZR-1, ZR-5, PG-9, PG-31, PG-1 and WW) were further analyzed for lipase activity using pH-Stat method at 55 °C. Plate assay is normally used for primary screening of lipase activity (Samad *et al.*, 1989). In this study Rhodamine B assay was used. Rhodamine B as-

Table 1 - Results of Biochemical tests and Rhodamine B plates were used for strain identifications and Rhodamine B plates and lipase activity assay.

Test	Organism					
	PG-1	PG-9	PG-31	ZR-1	ZR-5	WW
Gram reaction	+ ^a	+	-	+	+	+
Chains of cells	+	-	-	+	-	+
Motility	+	N	+/-	-	+/-	+
Spore position and shape	-	VX ^b	VX	VX	VX	VX
Swelling of cell body by spore	-	-	-	-	+/-	-
Growth at 50 °C	-	+	+	+	+	-
Growth in 10% NaCl	-	+	+	+	-	-
Anaerobic growth	-	+	+/-	+	+	-
Glucose	+	+	+	+	+	+
Galactose	+	-	+/-	-	-	+
Xylose	-	-	-	-	-	+
Manitol	-	+	+	+	+	-
Arabinose	-	-	+/-	+	-	-
Citrate	+	+	+	-	-	+
Indole	-	-	-	-	-	-
VP	-	-	-	-	-	-
Nitrate reduction	-	-	-	+	-	-
Casein hydrolysis	+	+	+	+	+	+
Starch hydrolysis	+	+	-	+	-	+
Catalase	+	+	+	+	+	+
Rhodamine B	+4 ^c	+4	+4	+4	+3	+4

^aThe results of other biochemical tests are presented as + and – signs indicating the presence or absence of that particular test, respectively.

^bV represents the oval shape and X shows the central/sub-central position of spores.

^cThe results of Rhodamine are presented as scores of +1 to +4 (+4 has the highest activity).

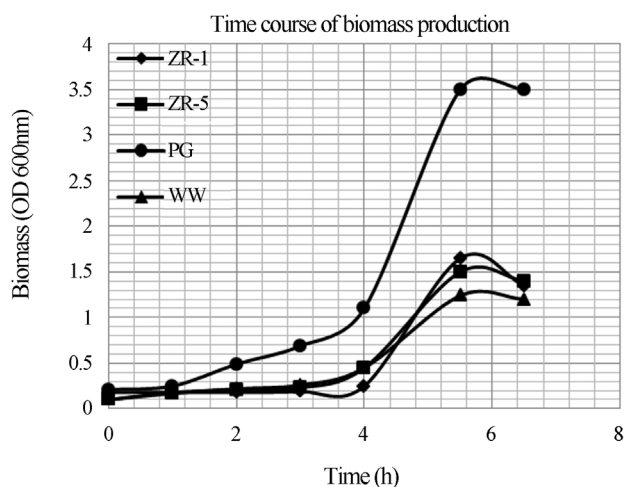


Figure 1 - Time course of growth and biomass production. Serial dilution was used for the OD higher than 1 and the points are means of three repeats.

say is a non-specific method for screening esterase activity and hence it cannot be used by itself to measure the lipase activity (Shu *et al.*, 2010). Plate assay for primary screening

of lipase activity are Thermophilic lipase activity of the selected strains grown on shake flask was measured by pH-Stat method using an auto-titrator at 55 °C. The activity measured after different incubation times (8-18 h with 2 h intervals) showed that ZR-1, ZR-5 and WW have the highest activity after 14 h of incubation, being respectively, 3.01, 2.33 and 1.87 U/mL (Figure 3). The optimum activity for PG-1, however, was 2.95 U/mL at 18 h after incubation (Figure 3). Other two strains, PG-9 and PG-31 did not show lipase activity using olive oil as substrate in pH-stat method at 55 °C.

The lipase activity was negligible before the late logarithmic phase however, after the end of log phase the lipase production was gradually increased (Figure 4). This could be due to consumption of carbon source available in the media which results in large biomass production. Limited amount of the carbon source forces the bacteria to use olive oil as an alternative carbon source for the production and maintenance of biomass which resulting more lipase production (Lima *et al.*, 2003).

The thermophilic lipase activity was analyzed at 55 °C next after fermentation process (37 °C, 220 rpm) for four selected strains. In comparison with shake flask the

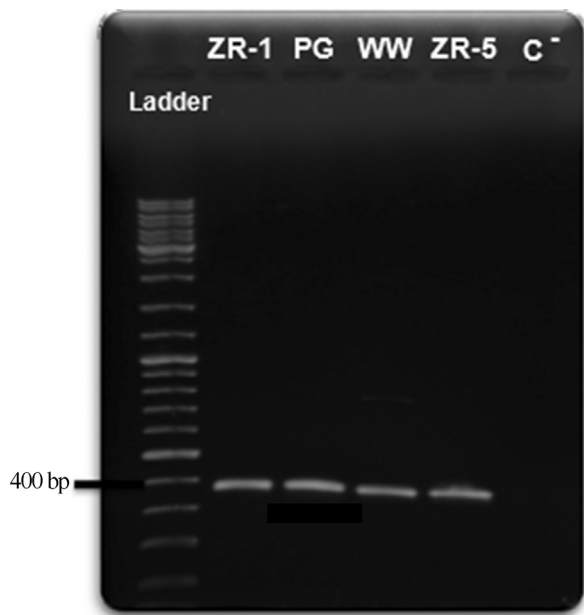


Figure 2 - Electrophoresis of PCR amplification of 16s rRNA on agarose gel (0.8% w/v). The lane on the left is the ladder (GeneRuler™ Mix, Fermentas) and the last is control. Clear bands at about 400 bp show the 16s rRNA gene from 4 samples.

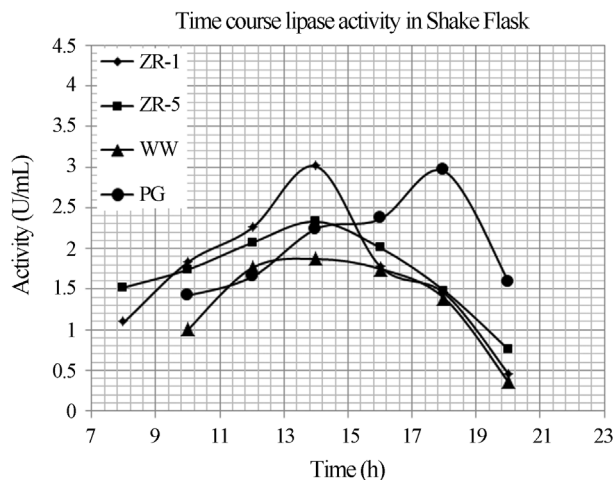


Figure 3 - Time course of lipase activity in shake flask method (37 °C, 220 rpm in LB with 2% v/v olive oil at pH 7.2). The lipase activity was measured using pH-Stat method (Titrand 902, Metrohm, Switzerland) and 12.5% v/v olive oil in gum Arabic (5% w/v) as substrate. The reaction was monitored for 10 min at 58 °C, pH 8 and 15 rpm. The points are means of three repeats.

lipase activity using fermentor for ZR-1, ZR-5, PG-1 and WW were increased by 40%, 51%, 15% and 49%, respectively (Figure 5). Fermentation also decreased the optimal time for maximum lipase production. The maximum lipase production time for WW and PG-1 was 13 h after incubation. The optimal lipase production time for ZR-1 was 12 h after incubation and there was no significant change in ZR-5 lipase activity after 14 h incubation time.

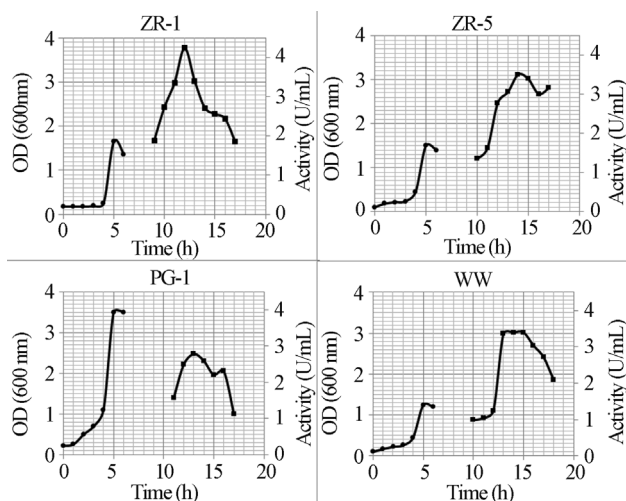


Figure 4 - Lipase production and growth phase relation. In all strains, maximum lipase production time is seen after logarithmic growth phase (l) biomass (n) lipase activity.

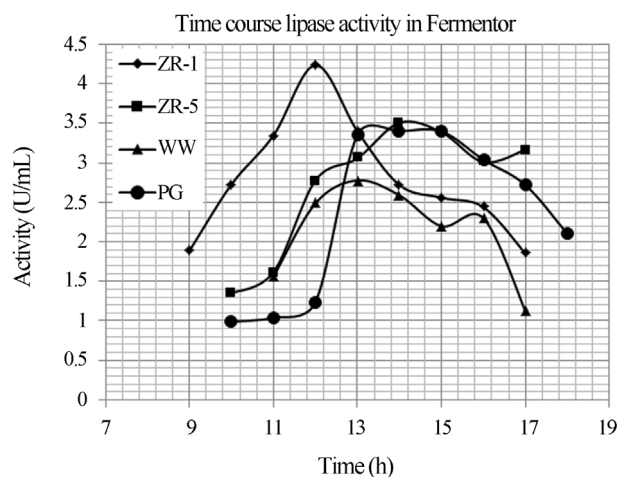


Figure 5 - Time course of lipase activity in Fermentation (37 °C, 220 rpm, DO 75% and aeration 4 l/min in LB with 2% v/v olive oil). The lipase activity was measured using pH-Stat method (Titrand 902, Metrohm, Switzerland) and 12.5% v/v olive oil in gum Arabic (5% w/v) as substrate. The reaction was monitored for 10 min at 58 °C, pH 8 and 15 rpm. The points are means of three repeats.

In order to improve the lipase production, a cultivation medium was designed. The strains were grown in shaking flask at 37 °C, 220 rpm for 72 h and the lipase activity was monitored at different time after incubation (24-48 hour with 6 h intervals). In comparison to LB medium containing 2% olive oil, the lipase activity for ZR-5, PG-1 and WW was increased by 4.55, 2.7 and 3.32, respectively. This medium although increased the activity but the growth rate was slower and the maximum activity was seen 30 h after incubation (Figure 6). Culture medium optimization generally improved the lipase productivity (Gupta *et al.*, 2007; Treichel *et al.*, 2010). This increase in lipase production varies from very low amount up to 50 fold depending

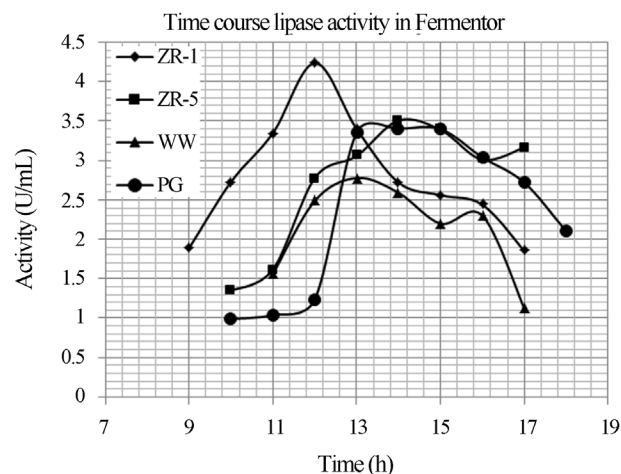


Figure 6 - Time course lipase activity in designed medium (37 °C, 220 rpm in LB with 2% v/v olive oil at pH 7.2). The lipase activity was measured using pH-Stat method (Titrand 902, Metrohm, Switzerland) and 12.5% v/v olive oil in gum Arabic (5% w/v) as substrate. The reaction was monitored for 10 min at 58 °C, pH 8 and 15 rpm. The points are means of three repeats.

on type organism and the composition of culture medium (Treichel *et al.*, 2010). Selection and optimization of each medium constituent is both costly and laborious (Teng and Xu, 2008). Therefore in this study, a medium was designed according to a literature review present on this subject. According to our literature review, it seems that the most significant factor in medium optimization to improve lipase productivity is type of carbon and nitrogen source (Kumari *et al.*, 2009; Lopez *et al.*, 2010; Lima *et al.*, 2003; Treichel *et al.*, 2010; Wang *et al.*, 2008). Therefore, this medium was designed so that the olive oil was the sole carbon source and ammonium sulfate was mineral nitrogen source. Different studies indicate that using oils such as plant oils, fatty acids and triglyceride as inducer or the sole carbon source increases the total lipase activity and productivity (Kumari *et al.*, 2009; Lopez *et al.*, 2010; Lima *et al.*, 2003; Treichel *et al.*, 2010; Wang *et al.*, 2008). This issue is also confirmed by our results. Using olive oil as the sole carbon source in this experiment, increase the activity of all stains except ZR-1. This increase for ZR-5 was maximum (4.55 fold) yielding an activity of 10.62 U/mL. According to the biochemical and molecular identification tests, ZR-1 and ZR-5, both belong to genus *Bacillus* and their behavior was so similar in other experiments but in this section, they show two completely different characterizations. Growth rate in designed medium for ZR-1 was too slow so that at the final point (72 h after incubation), the produced biomass was very small compared to ZR-5. It shows the significant effect of culture composition for each strain and directs us for more study in this part in future works.

The total activity of this strain was determined by lyophilization. The lyophilized form of crude lipase

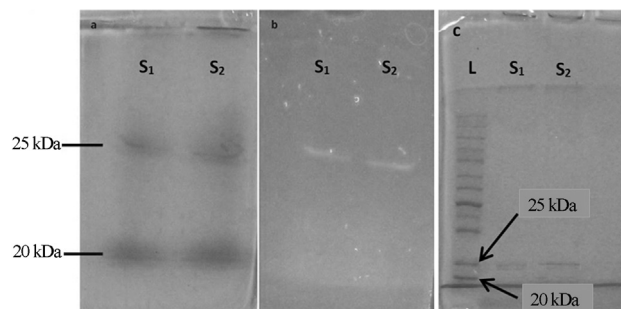


Figure 7 - SDS-PAGE (12% running gel) and Zymogram of *B. pumilus* (ZR-1) supernatant after lyophilization and dissolution in phosphate buffer. a) Native-PAGE of crude supernatant. b) Native-PAGE of crude supernatant under UV light at 350 nm. c) SDS-PAGE gel of semi-purified supernatant staining with Coomassie Brilliant Blue. The results indicated that the molecular weight of lipase from *B. pumilus* (ZR-1) is about 25 kDa. L; ladder SM0661 (Fermentas), S; sample.

showed high activity (12 kU/g) which is comparable with commercial lipases in the market. Further study for purification and determination of specific activity is required. Immobilization is another approach should be considered for industrial uses of this lipase.

Zymogram and electrophoresis

The samples for native-PAGE (15% homogenous gel) analysis were obtained at 30 h after incubation of ZR-5 in designed medium. Comparison of the stained gel with zymogram indicated that the extracellular lipase has a molecular weight about 25 kDa (Figure 7). According to biochemical and molecular identification (16s rRNA) test ZR-5 belongs to *B. pumilus* species. This species is a non-pathogenic natural soil microorganism and is found in a variety of foods raw materials and products. Colonizing around the root zone of plants prevents soil-borne fungal diseases and nematodes. It is also used for commercial production of cellulase. According to literature review, molecular weight of *B. pumilus* lipase is about 19.3 kDa which differ from our results (maybe because of different strain) and it is one of the smallest true lipases and they are very attractive in industry (Arpigny and Jaeger, 1999).

Conclusion

The aim of this work was studying new and native thermophilic lipase producing microorganisms. We could isolate four thermophilic extracellular lipase-producing strains. Further works such as fermentation and medium design increase the lipase activity of strains and among them, ZR-5 which belongs to *Bacillus pumilus* species showed the highest total activity after lyophilization of crude culture medium supernatant. Its characterizations, such as thermostability, alkalophilicity and acceptable total activity, introduce this lipase as good candidate for further studies (purification and immobilization) and show its potential for industrial applications.

Acknowledgments

This work was financially supported by research council of the Isfahan University of Medical Sciences, project number 390060, Isfahan, Iran.

References

- Adiguzel A, Ozkan H, Baris O, Inan K, Gulluce M, Sahin F (2009) Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *J Microbiol Methods* 79:321-328.
- Arpigny JL, Jaeger KE (1999) Bacterial lipolytic enzymes: classification and properties. *Biochem J* 343:177.
- Castro-Ochoa LD, Rodriguez-Gomez C, Valerio-Alfaro G, Oliart Ros R (2005) Screening, purification and characterization of the thermoalkalophilic lipase produced by *Bacillus thermo-levorans* CCR11. *Enzyme Microb Technol* 37:648-654.
- Claus D, Berkeley RCW (2011) Genus *Bacillus* Cohn 1872. In *Bergey's Manual of Systemic Bacteriology*, Sneath PHA, Mair NS, Sharpe NE, Holt JG (eds) pp 1105-1139. Williams and Wilkins: Baltimore
- Gupta N, Sahai V, Gupta R (2007) Alkaline lipase from a novel strain *Burkholderia multivorans*: Statistical medium optimization and production in a bioreactor. *Process Biochem* 42:518-526.
- Haba E, Bresco O, Ferrer C, Marques A, Busquets M, Manresa A (2000) Isolation of lipase-secreting bacteria by deploying used frying oil as selective substrate. *Enzyme Microb Technol* 26:40-44.
- Hasan F, Shah AA, Hameed A (2006) Industrial applications of microbial lipases. *Enzyme Microb Technol* 39:235-251.
- Hasan F, Shah AA, Hameed A (2007) Purification and characterization of a mesophilic lipase from *Bacillus subtilis* FH5 stable at high temperature and pH. *Acta Biol Hung* 58:115-132.
- Kouker G, Jaeger K-E (1987) Specific and Sensitive Plate Assay for Bacterial Lipases. *Appl Environ Microbiol* 53:211-213.
- Kumari A, Mahapatra P, Banerjee R (2009) Statistical optimization of culture conditions by response surface methodology for synthesis of lipase with *Enterobacter aerogenes*. *Braz Arch Biol Techn* 52:1349-1356.
- Lima VMG, Krieger N, Sarquis MIM, Mitchell DA, Ramos LP, Fontana JD (2003) Effect of nitrogen and carbon sources on lipase production by *Penicillium aurantiogriseum*. *Food Technol Biotechnol* 41:105-110.
- Lopez E, Deive FJ, Longo MA, Sanroman MA (2010) Culture Conditions and Investigation of Bioreactor Configurations for Lipase Production by *Rhizopus oryzae*. *Chem Eng Technol* 33:1023-1028.
- Messias JM, Da Costa BZ, De Lima VMG, Dekker RFH, Rezende MI, Krieger N, Barbosa AM (2009) Screening + a species for lipases: Production of lipase by *Botryosphaeria ribis* EC-01 grown on soybean oil and other carbon sources. *Enzyme Microb Technol* 45:426-431.
- Nguyen LN, Dao TT, Zivkovic T, Fehrholz M, Schafer W, Salomon S (2010) Enzymatic properties and expression patterns of five extracellular lipases of *Fusarium graminearum* in vitro. *Enzyme Microb Technol* 46:479-486.
- Nthangeni MB, Patterton HG, van Tonder A, Vergeer WP, Lithauer D (2001) Over-expression and properties of a purified recombinant *Bacillus licheniformis* lipase: a comparative report on *Bacillus* lipases. *Enzyme Microb Technol* 28:705-712.
- Rabbani M, Mir Mohammad Sadeghi H, Ani M, Goodarzvand Chegini K, Etemadifar Z, Moazen F (2009a) Cloning and nucleotide sequence of a lipase gene from a soil isolate. *Res Pharm Sci* 4:25-32.
- Rabbani M, Mirmohammadsadeghi H, Ani M, Chegini KG, Etemadifar Z, Moazen F (2009b) Functional expression of an alkaline lipase in *Escherichia coli*. *Ann Microbiol* 59:763-769.
- Samad MYA, Razak C, Salleh AB, Zin Wan Yunus WM, Ampon K, Basri M (1989) A plate assay for primary screening of lipase activity. *J Microbiol Methods* 9:51-56.
- Sheikh Abdul Hamid N, Zen HB, Tein OB, Halifah YM, Saari N, Bakar FA (2003) Screening and identification of extracellular lipase-producing thermophilic bacteria from a Malaysian hot spring. *World J Microbiol Biotechnol* 19:961-968.
- Shu ZY, Jiang H, Lin RF, Jiang YM, Lin L, Huang JZ (2010) Technical methods to improve yield, activity and stability in the development of microbial lipases. *J Mol Catal B: Enzym* 62:1-8.
- Teng Y, Xu Y (2008) Culture condition improvement for whole-cell lipase production in submerged fermentation by *Rhizopus chinensis* using statistical method. *Bioresour Technol* 99:3900-3907.
- Treichel H, de Oliveira D, Mazutti MA, Di Luccio M, Oliveira JV (2010) A review on microbial lipases production. *Food Bioprocess Tech* 3:182-196.
- Uttatree S, Winayanuwattikun P, Charoenpanich J (2010) Isolation and Characterization of a novel thermophilic-organic solvent stable lipase from *Acinetobacter baylyi*. *Appl Biochem Biotechnol* 162:1362-1376.
- Wang D, Xu Y, Shan T (2008) Effects of oils and oil-related substrates on the synthetic activity of membrane-bound lipase from *Rhizopus chinensis* and optimization of the lipase fermentation media. *Biochem Eng J* 41:30-37.
- Yuan B, Cai Y, Liao X, Yun L, Zhang F, Zhang D (2010) Isolation and identification of a cold-adapted lipase producing strain from decayed seeds of *Ginkgo biloba* L. and characterization of the lipase. *Afr J Biotech* 9:2661-2667.